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A mutation hotspot at the p14ARF splice site

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Germline mutations of *CDKN2A* that affect the p16INK4a transcript have been identified in numerous melanoma pedigrees worldwide. In the UK, over 50% of pedigrees with three or more cases of melanoma have been found to carry mutations of *CDKN2A*. Mutations that affect p14ARF exon 1β exclusively are very rare. This has led to the suggestion that it is p16INK4a and not p14ARF that plays the critical role in melanoma predisposition. We report the identification of a cluster of five different germline mutations at the p14ARF exon 1β splice donor site in melanoma pedigrees. All the five splice site variants showed evidence of being causal mutations. Three of the variants were demonstrated to result in aberrant splicing of the p14ARF mRNA, confirming their role in melanoma predisposition. No other point mutations were identified in the coding region of p14ARF. The p14ARF transcript of *CDKN2A* is clearly important in disease predisposition in a subset of melanoma pedigrees. Curiously, the only mutations so far reported to affect p14ARF exon 1β exclusively have been knockout mutations. Further investigation into the spectrum of mutations observed in this gene may help clarify the exact role of p14ARF in melanoma predisposition.
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The tumor suppressor gene *CDKN2A*, at chromosome 9p21, is the principal melanoma susceptibility gene identified to date. Mutations of *CDKN2A* have been shown to be involved in predisposition to disease in approximately 20% of melanoma pedigrees worldwide (Bishop *et al*., 2002).

The *CDKN2A* gene encodes the cyclin-dependant kinase inhibitor p16INK4a, which is thought to act through the Retinoblastoma (Rb) cell cycle control pathway. The p16INK4a protein controls passage through the G1 checkpoint of the cell cycle by inhibiting the phosphorylation of the retinoblastoma protein (Roussel, 1999). Evidence suggests that the p16INK4a protein may play a critical role in cell senescence, the acquired inability of cells to divide after a finite number of divisions (Alcorta *et al*., 1996; Hara *et al*., 1996; Bennett, 2003).

The *CDKN2A* locus also encodes p14ARF (Alternate Reading Frame). Unusually, p14ARF has a unique first exon (p14ARF exon 1β), but both transcripts utilize exons 2 and 3 of *CDKN2A,* although in different reading frames. The two proteins do not therefore share any homology at the amino-acid level; however, both appear to act as tumor suppressors (Quelle *et al*., 1995; Stone *et al*., 1995; Haber, 1997; Clurman and Groudine, 1998; Sharpless and DePinho, 1999). The p14ARF protein is thought to act via the p53 cell cycle control pathway, through interaction with the Human Double Minute 2 (HDM2) protein. This interaction prevents MDM2 from promoting the destruction of the p53 tumor suppressor protein, stabilizing p53 and resulting in cell cycle arrest at the G1/G2 phase (Sharpless and DePinho, 1999; Sherr, 2001).

The utilization of the same exon in different reading frames to encode two distinct proteins is extremely uncommon, and may even be unique in the human genome. The fact that the two proteins produced are tumor suppressor genes that act through different arms of the cell cycle control pathway makes the *CDKN2A* locus even more unusual.

To date, the majority of mutations identified in melanoma pedigrees have been observed in *CDKN2A* exons 1α and 2, affecting the p16INK4a transcript. This has led to the suggestion that p16INK4a and not p14ARF plays the critical role in predisposition to melanoma (Huot *et al*., 2002). Evidence of linkage to 9p21 in a number of pedigrees that do not carry p16INK4a mutations, however, suggests the presence of at least one other significant tumor suppressor gene at 9p21 (Dracopoli and Fountain, 1996; Hayward, 1996). In addition, the presence of a small number of reported

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deletions or mutations that specifically affect p14ARF (Randerson-Moor *et al*., 2001; Rizos *et al*., 2001; Hewitt *et al*., 2002) indicates that this transcript may play a significant role in melanoma predisposition.

In this study, a total of 184 DNA samples from 109 English pedigrees with two or more cases of melanoma were screened for sequence changes in p14ARF exon1β by sequencing analysis. A further 540 DNA samples from 270 international melanoma pedigrees from the Melanoma Genetics Consortium (GenoMEL www.genomel.org) were subsequently screened for p14ARF variants by DHPLC analysis. Ethical committee approval was obtained from multiregional and local ethics committees in England and all institutions involved internationally.

Five different variants were identified at the p14ARF 3′ splice site consensus sequence (Table 1). Three variants were initially identified in 109 English pedigrees screened by sequencing. A further two variants were identified in 270 international melanoma pedigrees screened by DHPLC. The five variants spanned consecutive bases in the p14ARF splice site consensus sequence from position '−2' to '+3', in relation to the p14ARF exon–intron boundary (Figure 1).

An additional variant, IVS1 + 56 T > C, was identified in two English pedigrees. This variant was situated in the intron at a distance from the p14ARF splice donor site, and does not affect the p14ARF splice site consensus sequence (Table 1). No other sequence variants were identified in p14ARF exon 1β.

None of the variants identified were detected in a panel of 338 control chromosomes screened by sequencing. All of the pedigrees investigated have previously been screened for mutations in *CDKN2A* exons 1α, 2 and 3, and exon 2 of *CDK4* (the p16INK4a-binding site) (Zuo *et al*., 1996; Harland *et al*., 1997; Goldstein *et al*., 2000; Harland *et al*., 2000). The p14ARF variants were observed in melanoma pedigrees in which no additional causal mutations had been identified. The evidence for the significance of the five variants is described below.

g.192 A > T: The predicted effect of this variant on the p14ARF splice site consensus sequence was severe, with a reduction in predicted likelihood of function from 95 to 52% (Table 1). Segregation of the variant was compatible with an association with disease, although this could not be established conclusively due to limited pedigree size (Figure 2). Samples were not available for RNA extraction and therefore RT–PCR could not be used to investigate the effect of this variant on splicing.

g.193 G > C: RT–PCR of RNA derived from a sample positive for the variant g.193 G > C revealed aberrant splicing of the mRNA. The aberrant splicing appeared to utilize a cryptic splice donor site at position '−12' in the p14ARF 5′UTR, and an acceptor in exon 2 at base 463 of *CDKN2A*, relative to the p14ARF 'ATG' start codon (Figure 3). The exonic sequence variant (G > C) was not detected in the wild-type RT–PCR band, suggesting that the variant allele does not produce normally spliced mRNA. The segregation of the g.193 variant was not, however, entirely consistent with an association with disease, with an affected grandparent not carrying this mutation (Figure 2).

g.193 + 1 G > A: This variant had been independently identified in a Dutch melanoma pedigree by sequencing and was subsequently detected by DHPLC analysis in this study. RT–PCR of RNA, derived from a sample positive for the variant g.193 + 1 G > A, showed exactly the same pattern of aberrant splicing as described for variant g.193 G > C (Figure 3). The variant was shown to segregate with disease (Figure 2).

g.193 + 2 T > C: The g.193 + 2 variant was identified by DHPLC screening in an American melanoma pedigree. This variant is currently under investigation in the US, where it has been found to show evidence of association with disease (J Struewing, manuscript in preparation).

g.193 + 3 A > G: RT–PCR of RNA from a sample positive for the g.193 + 3 variant showed evidence of aberrant splicing. The aberrant splicing appeared to employ a cryptic donor site at base 78 of p14ARF exon 1β. The p14ARF mRNA was spliced from this cryptic donor site to a splice acceptor in *CDKN2A* exon 3 (Figure 3). Segregation of the g.193 + 3 A > G variant was compatible with an association with disease, although pedigree size was small (Figure 2).

g.193 + 56: The g.193 + 56 variant was not predicted to affect the p14ARF splice site consensus sequence, nor was it predicted to create or enhance a cryptic splice site (Table 1). Segregation with disease could not be investigated for this variant due to lack of available samples. RT–PCR of RNA derived from a sample positive for the g.193 + 56 variant did not demonstrate any evidence of aberrant splicing.

Table 1 Summary of p14ARF exon 1β variants identified

Location[a]	*Base change*	*Splice site position*	*Predicted likelihood of functional impairment*[b]	*Number of pedigrees*	*Origin of pedigree*
g.192	A > T	−2	95 > 52%	1	England
g.193	G > C	−1	95 > 29%	1	England
g.193 + 1	G > A	+1	95 > 0%	1	Netherlands
g.193 + 2	T > C	+2	95 > 0%	1	USA
g.193 + 3	A > G	+3	95 > 75%	1	England
g.193 + 56	T > C	+56	None	2	England

[a]Locations of base changes are given relative to the 'ATG' initiation codon of p14ARF, following the standard nomenclature for sequence variation (den Dunnen and Antonarakis, 2001). [b]Predicted effect on the likelihood of splice donor function being impaired was calculated using the 'NNSPLICE0.9' neural network splice site prediction tool (Reese *et al*., 1997). The percentage score gives an indication of the probability that the splice site will be functional, based on the surrounding consensus sequence (http://www.fruitfly.org/seq_tools/splice.html)

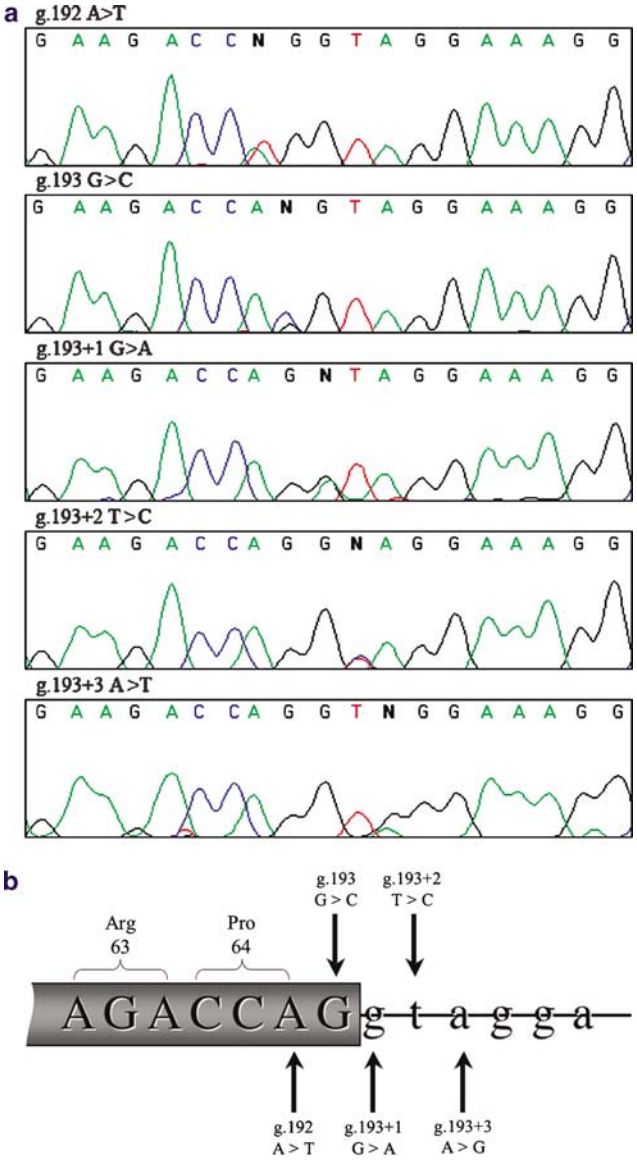

Figure 1 (**a**) The identification of five sequence variants at consecutive bases across the p14ARF exon 1β splice donor site by direct sequencing and DHPLC analysis. '**N**' indicates the location of the base changes observed. (**b**) Schematic representation of the p14ARF exon 1β splice donor site, showing the location of the five variants identified. Sequencing analysis of p14ARF exon 1β was carried out in 109 English melanoma pedigrees. Exon 1β was amplified using a standard PCR protocol (Harland *et al*., 1997), with the primers p14-1F – CAC CTC TGG TGC CAA AGG GC and p14-1R – CCT AGC CTG GGC TAG AGA CG. Sequencing reactions were carried out directly on the exon 1β PCR products, using the above primers, and were analysed on an ABI 3100 Sequencer (Perkin-Elmer). DHPLC analysis in 270 international melanoma pedigrees was carried out at 61, 65 and 69°C, as determined by the 'DHPLC Melt' program (Jones *et al*., 1999), using a Transgenomic WAVE Nucleic Acid Fragment Analysis system and DNASep column (Transgenomic, Crewe, UK)

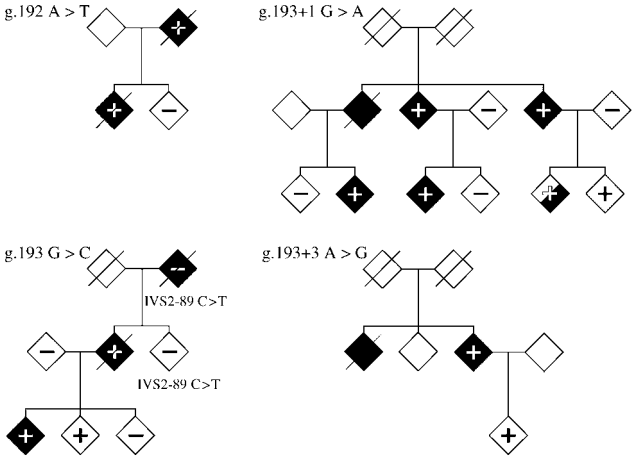

Figure 2 Segregation of the p14ARF splice site variants with disease. Pedigree diagrams have been simplified and adjusted to protect the identities of families. Solid symbols represent individuals with cutaneous melanoma. The half-filled symbol represents an individual with a brain tumor at age 15. The presence of each p14ARF splice variant is indicated by (+), absence of the variant is indicated by (−), other individuals have not been tested. The presence of the *CDKN2A* intronic variant IVS2-89 in two individuals from the p14ARF g.193 G>C pedigree is also indicated

We have identified five different sequence variants at the p14ARF splice donor consensus sequence. Aberrant splicing of mRNA was demonstrated, using RT–PCR, for three of the variants identified (g.193, g.193 + 1 and g.193 + 3), suggesting that they may be causal mutations, predisposing to melanoma.

The variants g.193 and g.193 + 1 were shown to result in exactly the same pattern of aberrant splicing. In each case, a cryptic splice site in the 5′UTR of p14ARF was activated, and spliced to a cryptic acceptor in exon 2 of *CDKN2A*. Any translated protein would clearly not be functional. It is interesting to note the occurrence of a brain tumour in a mutation-positive individual from the g.193 + 1 pedigree (Figure 2), as a link between the deletion of one copy of p14ARF exon 1β and neural system tumours has previously been reported in an English melanoma pedigree (Randerson-Moor *et al*., 2001).

An affected grandparent in the g.193 melanoma pedigree, whose melanoma status was confirmed by hospital records, was found not to carry this particular variant (Figure 2). This may represent a sporadic melanoma case, or predisposition to disease in this individual may be due to an additional genetic variant. Interestingly, this individual carries the intronic CDKN2A variant IVS2-89 C>T (Harland *et al*., 2005). This variant had previously been regarded as a non-causal polymorphism, due to lack of segregation with disease in this pedigree. The status of the IVS2-89 variant may now have to be re-examined. The g.193 variant has previously been identified in an unconnected English melanoma pedigree, where it has been shown to segregate with disease (Hewitt *et al*., 2002). This, together with the evidence of aberrant splicing reported here, indicates that g.193 G>C is likely to be a causal variant.

The variant g.193 + 3 also showed evidence of aberrant mRNA splicing. Although the aberrant splicing observed with g.193 + 3 was different from that

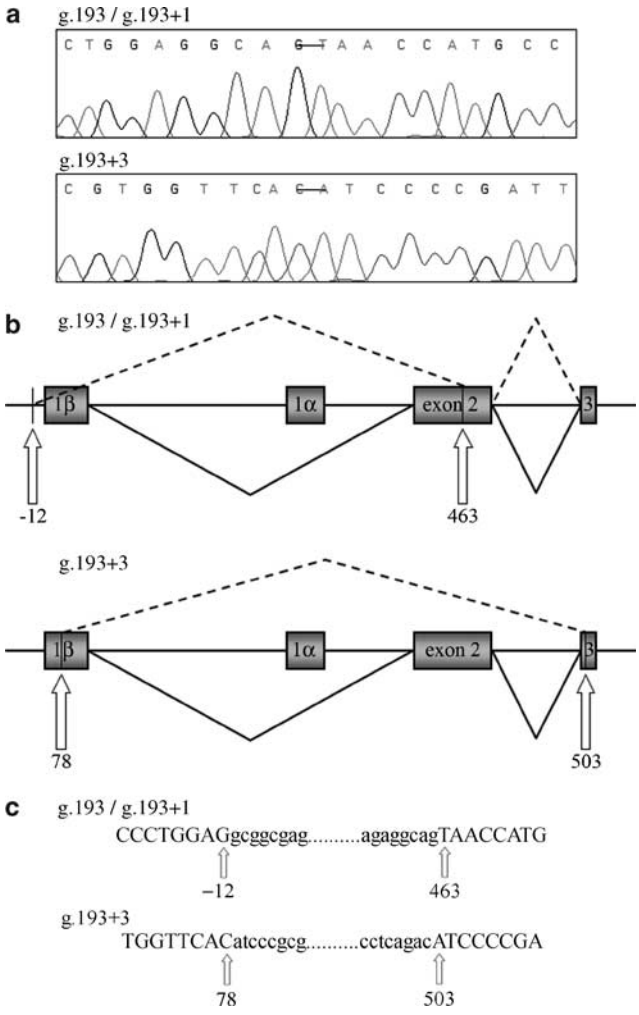

Figure 3 (**a**) Chromatograms showing the nucleotide sequence of aberrant RT–PCR products around the cryptic splice sites identified. The solid line at the center of the nucleotide sequence indicates the location of the aberrant splicing event. Total RNA was extracted from B-lymphoblastoid cell lines using the Qiagen RNeasy Midi Kit. cDNA was transcribed using the Advantage RT for PCR kit (Clontech). The p14ARF mRNA transcript was amplified using a forward primer in the p14ARF exon 1β 5′UTR (p14 1F – CAC CTC TGG TGC CAA AGG GC) and a reverse primer in the *CDKN2A* 3′UTR (RT 3R – CCT GTA GGA CCT TCG GTG AC), using a standard PCR protocol (Harland *et al*., 1997). The aberrantly sized bands produced by RT–PCR were gel purified (Qiaquick Gel Extraction Kit, Qiagen) and re-amplified prior to sequencing. (**b**) Schematic representation of splicing of the p14ARF transcript. Standard, wild-type splicing is indicated by solid lines; aberrant splicing is indicated by dashed lines. The locations of the cryptic splice sites, relative to the first coding nucleotide of p14ARF, are arrowed. (**c**) Nucleotide sequence around the p14ARF cryptic splice sites. Nucleotides shown in upper case are retained in the aberrantly spliced mRNA, nucleotides in lower case are spliced out. The locations of the aberrant splice sites relative to the first coding nucleotide of p14ARF are indicated

seen with variants g.193 and g.193 + 1, the resulting mRNA, if translated, would also be predicted to give rise to a nonfunctional p14ARF protein. We were unable to investigate splicing in variant g.192, but the position of the variant and its predicted effect on the p14ARF splice consensus sequence suggest that it too may be capable of disrupting the splicing of the p14ARF transcript.

It should be noted that the cDNAs used in this investigation were derived from B-lymphoblastoid cell lines. Tissue-specific splicing has previously been demonstrated in *CDKN2A* (Robertson and Jones, 1999), and it is conceivable that the effect of any of the p14ARF splice site mutations on the protein at the site of melanogenesis may be different from that observed in lymphocytes.

The aberrantly spliced mRNAs observed in this study do not use a standard GT-AG splice site consensus sequence. Almost all introns start with GT and end with AG (Mount, 1982). However, the splicing variant observed with g.193 + 3 appears to follow the less common AT to AC dinucleotide rule (Shapiro and Senapathy, 1987; Tarn and Steitz, 1996), whereas the variant observed with g.193 and g.193 + 1 appears to make use of GC to AG terminal dinucleotides, which are also compatible with splicing (Parker and Siliciano, 1993; Zaphiropoulos, 1998) (Figure 3c).

It has been demonstrated that artifacts resembling aberrantly spliced RNA transcripts can be generated during RT–PCR. These artifacts consist of segments of exons joined at non-consensus splice sites, and are thought to arise through the misalignment of a prematurely terminated strand with a homologous region in the other strand during amplification (Meyerhans *et al*., 1990; Paabo *et al*., 1990; Zaphiropoulos, 1998). It is unlikely, however, that the splicing variants observed in this study represent PCR artifacts. Identical splicing events were observed in repeated amplifications, and, in the case of g193 and g193 + 1, the same splicing event is seen in different pedigrees. PCR artifacts were not observed using RNA derived from wild-type samples or from the g.193 + 56 variant. In addition, the splice site consensus sequences observed are known to be compatible with splicing. The variants described here are therefore likely to signify actual splicing events.

Although the predicted effect of these aberrant splicing events on the translated p14ARF protein is severe, it is possible that it is the instability of the aberrant splice products that is the critical factor in predisposition to disease. There is evidence that specific systems of nuclear degradation target and degrade aberrant pre-mRNA molecules before they can be translated into protein (Hilleren and Parker, 2003). Whether through mRNA instability, or through translation of a nonfunctional protein, the effect on p14ARF function is likely to be the same. In each case, the aberrant splicing may well completely abolish the contribution of the mutant allele, reducing the amount of wild-type protein produced to below a threshold level and impairing the function of p14ARF in cell cycle control and senescence.

Mutations that specifically affect the p14ARF transcript appear to be a rare cause of predisposition to melanoma. In addition to splice site variants, there are two other reported germline anomalies abrogating p14ARF in melanoma families. An exon 1β-specific

deletion of p14ARF in an English melanoma-neural system tumor pedigree (Randerson-Moor *et al*., 2001) and a 16 base pair insertion, resulting in a frameshift in a Spanish melanoma pedigree (Rizos *et al*., 2001). Each of these examples can be expected to completely abolish the contribution of the mutant p14ARF allele, as is predicted for the splice site variants described here.

The p14ARF transcript therefore shows an unusual spectrum of mutations. Five different mutations have been observed at the exon 1β spice donor site. Furthermore, no additional point mutations were identified in the remainder of exon 1β. It is conceivable that the complete knockout of an entire allele may be required in order for p14ARF to contribute to predisposition independently of p16INK4a.

This study establishes that mutations of p14ARF, specifically splice site variants, are causal in a subset of melanoma pedigrees independently of p16INK4a. The p14ARF transcript of *CDKN2A* is clearly the third high-penetrance melanoma susceptibility gene.

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